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CXCL12, CXCR4 AND IL8 GENETIC POLYMORPHYSM IN THE PROGNOSIS OF HUMAN GASTRIC CANCER.

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RIASSUNTO

Presupposti dello studio: La prognosi del cancro gastrico non è migliorata in maniera significativa negli ultimi anni. Attualmente i due principali fattori prognostici sono rappresentati dallo stadio TNM alla diagnosi e dalla possibilità di ottenere un intervento chirurgico apparentemente radicale.

Scopo dello studio: Individuare nuovi fattori prognostici indipendenti che permettano di migliorare la stratificazione del rischio di decesso per carcinoma gastrico e selezionare quei soggetti che potrebbero beneficiare di una terapia adiuvante dal momento che la stadiazione TNM presenta un certo grado di incertezza soprattutto negli stadi intermedi e che, nonostante una chirurgia apparentemente radicale, la prognosi rimane insoddisfacente in una significativa percentuale di casi. Si è studiato il microambiente tumorale per le crescenti evidenze indicanti un ruolo fondamentale del microambiente tumorale nei processi di proliferazione, angiogenesi e metastatizzazione.

Materiali e metodi: Sono stati arruolati 333 soggetti sottoposti ad intervento chirurgico per carcinoma gastrico (stadi I- IV) a partire dal 1991. Ogni soggetto è stato sottoposto ad un prelievo di sangue venoso periferico nel pre-operatorio dalle cui PBMC è stato estratto il DNA (germline) per la discriminazione allelica. Lo studio ha esaminato un set di polimorfismi di tre diversi geni (codificanti per le citochine CXCL12 e IL8 e per il recettore CXCR4) e la presente tesi riporta dei risultati parziali che riguardano i seguenti tre polimorfismi: rs1801157, rs2228014 e rs4073.

Risultati: L'analisi di sopravvivenza non ha riportato risultati statisticamente significativi (p-value>0,05) nei modelli genetici allelico, dominante e recessivo. Per quanto riguarda i dati anatomo-patologici esaminati non è stata individuata una correlazione statisticamente significativa (p-value>0,05) tra i polimorfismi e grading, infiltrazione linfatica e venosa nei tre modelli genetici. È stata, invece, evidenziata un'associazione debolmente significativa (p-value=0,049) tra i genotipi A/A e A/T del polimorfismo rs4073 del gene codificante per IL8 e il coinvolgimento linfonodale nel modello dominante. Alla regressione logistica con variabile dipendente il coinvolgimento linfonodale: odds ratio=0,602, p-value=0,050, I.C.95%=0,363-0.999. All'analisi multivariata il polimorfismo non è risultato essere un fattore indipendente dal parametro T della stadiazione TNM (odds ratio=0,663; I.C. 95%=0,361-1,215; p-value:0,184).

Conclusioni: Questo lavoro suggerisce che vi siano delle evidenze interessanti a favore dell'associazione dell'allele T del polimorfismo rs4073 e la riduzione del rischio di metastasi linfonodali, ma che non possano essere ritenute conclusive. Sono pertanto necessari ulteriori studi allo scopo di analizzare ulteriori polimorfismi del gene per IL8 che potrebbero spiegare, con un livello di significatività più consistente, l'associazione tra la proteina e il coinvolgimento linfonodale.

SUMMARY

Background: In the last few years gastric cancer's prognosis hasn't improved. At the present the two main prognostic factors are the TNM staging classification and surgery with radical intent.

Objective: To identify independent prognostic factors to improve the risk stratification of gastric cancer's death and to select patients at high risk to be submitted to adjuvant treatment because the TNM classification presents uncertainty primarily in the intermediate stages and, although an apparently radical surgery, gastric cancer prognosis remains poor. The study is based on tumor microenvironment because the are many recent evidences that underline its fundamental role in tumor growth, angiogenesis and metastasis.

Materials and Methods: 333 patients affected with gastric cancer at different TNM stages (I- IV) of disease who underwent radical surgery from 1991. Before surgery a sample of peripheral blood was withdrawn from each patient. From each sample PBMC were used for DNA (germline) isolation for allelic discrimination assay. This study evaluated a set of polymorphisms of three different genes coding for CXCL12 and IL8 (cytokines) and CXCR4 (receptor) and the present work shows the partial results concerning the following three polymorphisms: rs1801157, rs2228014 e rs4073.

Results: Survival analysis reported no statistically significant results (p-value>0,05) in allelic, dominant and recessive genetic models. As regards the data anatomo-pathological, a statistically significant correlation between polymorphisms and grading and lymphatic and venous infiltration in the three genetic models was not found (p-value>0,05). Instead, a weakly association (p-value=0,049) between genotypes A/A and A/T of rs4073 of the gene encoding for IL8 and lymph node involvement was found in the dominant model. At the logistic regression with lymph node involvement as dependent variable: odds ratio=0,602, p-value=0,050, I.C.95%=0,363-0.999. At the multivariate analysis the polymorphism wasn't found to be an independent factor of T parameter of TNM staging (odds ratio=0,663; I.C. 95%=0,361-1,215; p-value:0,184).

Conclusions: The results of this thesis suggest that there are interesting evidences in favour of the association between the allele T of rs4073 and the decreased risk of lymph node metastasis but that these can't be considered conclusive. New studies are needed to analyze additional polymorphisms of the gene encoding for IL8 because these can explain the association between the protein and the lymph node involvement with a more consistent level of significance.

1. INTRODUCTION

1.1 Epidemiology

Despite a major decline in incidence and mortality over several decades, stomach cancer is still the fourth most common cancer and the second most common cause of cancer death in the world. There is a 10-fold variation in incidence between populations at the highest and lowest risk. The global distribution of gastric cancer varies substantially across geographical regions which illustrate the multitude of factors that are associated with the incidence, survival and mortality of the disease [1,2]. The Asian countries account for the majority of the world's cases while Europe and the Americas combined makeup less than a quarter of the world disease burden. Seventy-three percent of gastric cancer cases are diagnosed in Asia; almost 50% of the world's cases are diagnosed in China alone. Europe accounts for an additional 15% and Central and South America contribute 7% of the global burden [3,4]. Within these global regions, there is further variability as to which populations are more greatly affected. The incidence rate in men is double that of women and incidence increases with age with a peak incidence between the fifth and the seventh decades. Even within the same geographic region certain ethnic groups have significantly higher risk of disease. Within the United States, Hispanics, African Americans, and Native Americans are more frequently affected than Caucasian Americans [3]. However, ethnic predisposition cannot be considered alone since socioeconomic status also impacts disease incidence. In the last decades a decline in incidence was observed and probably due to improved nutrition, food preservation (great intake of fresh fruits and vegetables), better prevention, earlier diagnosis and treatment. Although the disease still carries a poor prognosis.

In Italy we register a high variability in incidence rate and mortality for gastric cancer with a maximum in Toscana, Emilia Romagna, Friuli and Marche, and a minimum in Campania e Puglia [5].

1.2 Histologic classification

Several classification systems exist to define gastric cancer but the most frequently used is the Lauren classification. The Lauren classification defines two main histologic subtypes: Intestinal type and diffuse type, plus indeterminate type as uncommon variant [6]. Each subtype represents distinct clinical and epidemiologic characteristics. There are rare cases of gastric carcinomas that display features of both histologic subtypes. The morphologic differences between the two subtypes are related to intercellular adhesion molecules, which are preserved in intestinal type disease and defective in diffuse gastric carcinoma. The relative frequencies are approximately 54% for intestinal type, 32% for the diffuse type, and 15% for the indeterminate type [7]. There are indications that the diffuse type gastric carcinoma is more often seen in female and young individuals [8,9], while the intestinal type adenocarcinoma is more often associated with intestinal metaplasia and Helicobacter pylori infection [10,11].

The 2010 WHO classification recognizes four major histologic patterns of gastric cancers: tubular, papillary, mucinous and poorly cohesive (including signet ring cell carcinoma), plus uncommon histologic variants [12]. The classification is based on the predominant histologic pattern of the carcinoma which often co-exists with less dominant elements of other histologic patterns. Tubular adenocarcinoma is the most common histologic type of early gastric carcinoma. It tends to form polypoid or fungating masses grossly, and histologically demonstrates irregularly distended, fused or branching tubules of various sizes, often with intraluminal mucus, nuclear and inflammatory debris. Papillary adenocarcinoma is another common histologic variant often seen in early gastric carcinoma. It tends to affect older people, occur in the proximal stomach, and is frequently associated with liver metastasis and a higher rate of lymph node involvement. Histologically, it is characterized by epithelial projections scaffolded by a central fibrovascular core. Mucinous adenocarcinoma accounts for 10% of gastric carcinoma. Histologically it is characterized by extracellular mucinous pools which constitute at least 50% of tumor volume. The tumor cells can form glandular architecture and irregular cell clusters, with occasional scattered signet ring cells floating in the mucinous pools. Signet ring cell carcinoma and other poorly cohesive carcinomas are often composed of a mixture of signet ring cells and non-signet ring cells. Poorly cohesive non-signet ring tumor cells are those that morphologically resemble histiocytes, lymphocytes, and plasma

cells. Those tumor cells can form irregular microtrebaculae or lace-like abortive glands, often accompanied by marked desmoplasia in the gastric wall and with a grossly depressed or ulcerated surface. When it occurs at the antropyloric region with serosal involvement, the carcinoma tends to have lymphovascular invasion and lymph node metastasis. Because signet ring cell and other poorly cohesive carcinomas at antroplyoric region have a propensity to invade duodenum via submucosal and subserosal routes including subserosal and submucosal lymphatic spaces, special attention needs to be paid to those routes when a distal margin frozen section is requested at the time of surgical resection. Special stains such as cytokeratin immunohistochemistry can help detect morphologically occult signet ring cells in the lamina propria. One important differential diagnosis of neoplastic signet ring cells in gastric mucosa is benign pseudo-signet ring cells which can remarkably mimic signet ring cell carcinoma. Those pseudo-signet ring cells sometimes can demonstrate cytological atypia, even with mitoses. However, those pseudo-signet ring cells do not reveal invasive pattern with reticulin stain which highlights pseudo-signet ring cells confined within basement membrane with intact acinar architecture [13]. In addition to the above four major histologic subtypes, WHO classification also endorses other uncommon histologic variants, such as adenosquamous carcinoma, squamous carcinoma, hepatoid adenocarcinoma, carcinoma with lymphoid stroma, choriocarcinoma, parietal cell carcinoma, malignant rhabdoid tumor, mucoepidermoid carcinoma, paneth cell carcinoma, undifferentiated carcinoma, mixed adeno-neuroendocrine carcinoma, endodermal sinus tumor, embryonal carcinoma, pure gastric yolk sac tumor and oncocytic adenocarcinoma, all listed in Table 1, with Lauren's classification for comparison. Gastric carcinoma with lymphoid stroma (medullary carcinoma) is one of the uncommon subtypes. It occurs more commonly in proximal stomach and generally follows a less aggressive clinical course. Micropapillary carcinoma of stomach is a newly recognized histologic variant characterized by small papillary clusters of tumor cells without a distinct fibrovascular core. Micropapillary carcinoma of stomach, as its counterpart at other organs, tends to form endolymphatic tumor emboli and metastasize to lymph nodes.

Table 1 Gastric adenocarcinoma classification systems

WHO (2010)	Lauren (1965)
Papillary adenocarcinoma Tubular adenocarcinoma Mucinous adenocarcinoma	Intestinal type
Signet-ring cell carcinoma And other poorly cohesive carcinoma	Diffuse type
Mixed carcinoma	Indeterminate type
Adenosquamous carcinoma	
Squamous cell carcinoma	
Hepatoid adenocarcinoma	
Carcinoma with lymphoid stroma	
Choriocarcinoma	
Carcinosarcoma	
Parietal cell carcinoma	
Malignant rhabdoid tumor	
Mucoepidermoid carcinoma	
Paneth cell carcinoma	
Undifferentiated carcinoma	
Mixed adeno-neuroendocrine carcinoma	
Endodermal sinus tumor	
Embryonal carcinoma	
Pure gastric yolk sac tumor	
Oncocytic adenocarcinoma	

1.3 Classification based on anatomic location

For the classification based on anatomic location, difficulty often arises when the tumor is located at proximal stomach or cardia, especially when the tumor also involves gastroesophageal junction (GEJ). It is not only because there are shared histologic features and immunophenotypes between the inflamed gastric cardiac mucosa due to Helicobacter infection and the metaplastic columnar epithelium-lined distal esophageal mucosa secondary to reflux disease, but also because there is no universal consensus regarding the anatomic definition of gastric cardia [14,15]. Several classifications were proposed in order to address this issue. The scheme endorsed by the International Gastric Cancer Association separates gastric cancers into type I, type II and type III, to represent the tumors at distal esophagus, at cardia and at the stomach distal to cardia, respectively [16]. This classification, however, has not clearly defined the criteria for each of these anatomic locations. Most recently, the 7th Edition of the TNM classification by American Joint Committee on Cancer (AJCC) has simplified the classification of the carcinoma at proximal stomach based on the location of tumor epicenter and the presence or absence of GEJ involvement [17]. The tumor is to be stage grouped as esophageal carcinoma if its epicenter is in the lower thoracic esophagus or GEJ, or within the proximal 5 cm of

stomach (i.e., cardia) with the tumor mass extending into GEJ or distal esophagus. If the epicenter is >5 cm distal to the GEJ, or within 5 cm of GEJ but does not extend into GEJ or esophagus, it is stage grouped as gastric carcinoma [17]. This classification, although easy for pathologists to follow, could still face some challenges. For example, a bulky gastric cardiac cancer with its epicenter 4 cm below GEJ will still be diagnosed and classified as an esophageal tumor if the proximal end of tumor extends into GEJ by only 0.5 cm (even if the distal end of tumor is 4 cm from the epicenter extending into the stomach). For the operating surgeon who sees the tumor in situ, it may be difficult for him or her to accept this tumor as an esophageal cancer. In addition, a recent retrospective study by Huang et al. shows that cardiac carcinoma involving GEJ or distal esophagus is more appropriately classified and staged as gastric rather than esophageal cancers, at least in the Chinese population [18]. In that study, cardiac carcinomas were staged according to the depth of invasion, status of positive lymph nodes and distant metastasis, as both gastric and esophageal tumors. When the tumor stage is studied and compared with cumulative survival, the findings support that it is more appropriately to group and stage cardiac cancers as stomach in origin [18]. To better separate gastric cardiac carcinoma from esophageal or GEJ malignancy, more studies are apparently needed, such as a larger patient sample, molecular profiling of the tumor, clinical follow up data, and defining the tumor location after neoadjuvant therapy as to determine whether the initially bulky tumor was more "gastric" or more "GEJ/esophagus" in origin.

1.4 Early and advanced gastric carcinoma

Early gastric carcinoma is defined as invasive carcinoma confined to mucosa and/or submucosa, with or without lymph node metastases, irrespective of the tumor size [19]. Most early gastric carcinomas are small, measuring 2 to 5 cm in size, and often located at lesser curvature around angularis. Some early gastric carcinoma can be multifocal, often indicative of a worse prognosis. Grossly, early gastric carcinoma is divided into Type I for the tumor with protruding growth, Type II with superficial growth, Type III with excavating growth, and Type IV for infiltrating growth with lateral spreading. Type II tumor is further divided to IIa (elevated), IIb (flat) and IIc (depressed), as proposed by the Japanese Endoscopic Society [20]. A more recent Paris classification has endorsed three gross patterns for superficial neoplastic lesions in gastrointestinal tract. Grossly and

endoscopically, the tumor is classified as Type 0-I for polypoid growth (which is subcategorized to 0-Ip for pedunculated growth and 0-Is for sessile growth), Type 0-II for nonpolypoid growth (which is subcategorized into Type 0-IIa for slightly elevated growth, Type 0-IIb for flat growth, and Type 0-IIc for slightly depressed growth), and Type 0-III for excavated growth [21]. Histologically, the most common forms of early gastric carcinoma are well differentiated, mostly with tubular and papillary architecture. The distinction between well-differentiated carcinoma and high grade dysplasia or carcinoma in situ can be challenging when only mucosal tissue is available for histologic assessment. Intramucosal invasion may not be as easily confirmed as an invasive carcinoma into submucosa where stromal desmoplasia is usually evident. The distinction between intramucosal carcinoma and carcinoma in situ or high grade dysplasia is important, as the intramucosal carcinoma of stomach, unlike the intramucosal carcinoma in the colon, does metastasize. Generally, the useful histologic features of intramucosal invasion are single tumor cells in the lamina propria and significantly fused neoplastic glands of various sizes. The prognosis of early gastric carcinoma is excellent, with a 5 years survival rate as high as 90% [22]. In contrast, the advanced gastric carcinoma which invades into muscularispropria or beyond carries a much worse prognosis, with a 5 years survival rate at about 60% or less [23]. The gross appearance of advanced gastric carcinomas can be exophytic, ulcerated, infiltrative or combined. Based on Borrmann's classification, the gross appearance of advanced gastric carcinomas can be divided into type I for polypoid growth, type II for fungating growth, type III for ulcerating growth, and type IV for diffusely infiltrating growth which is also referred to as linitisplastica in signet ring cell carcinoma when most of gastric wall is involved by infiltrating tumor cells. Histologically, advanced gastric carcinoma often demonstrates marked architectural and cytological heterogeneity, with several co-existing histologic growth patterns. The distinction between early and advanced gastric carcinoma before resection is clinically important because it helps decide if a neoadjuvant (pre-operative) therapy which has shown to improve disease free survival and overall survival [24,25] is warranted. While the macroscopic appearance is informative, the most accurate pre-operative staging information is generally obtained with endoscopic ultrasonography (EUS) and computer tomography (CT) [26].

1.5 Risk factors for gastric cancer

Risk factors for gastric cancer can be environmental and lifestyle related or genetic.

Helicobacter pylori (H. pylori) is the primary environmental carcinogen as this ancient bacterium has a complex ability to interact with its human host. Smoking and salt are strong independent risk factors for gastric cancer whereas alcohol is only a risk when it is heavily consumed. Red meat and high fat increase the risk of gastric cancer however fresh fruits, vegetables (allium family) and certain micronutrients (selenium, vitamin C) reduce the risk, with evidence lacking for fish, coffee and tea. Foods that inhibit H. pylori viability, colonization and infection may reduce cancer risk. Obesity is increasingly recognized as a contributory factor in gastric cardia carcinogenesis. Therefore, modest daily physical activities can be protective against cancer. Foundry workers are at risk for developing gastric cancer with dust iron being an important cause. Other risk factors include Epstein-Barr virus (EBV), possibly JC virus and radiation but the effects of these are likely to remain small [27].

Early studies revealed that gastric cancer was less common in patients with blood group 0, but was frequently associated with blood group A which increases the risk by 16-20%. A positive family history of gastric cancer has been associated with an increased (~three-fold) risk of gastric cancer. Interestingly, subjects with both a positive family history and infection with cagA-positive H. pylori strains had a 16-fold increased risk of non-cardia gastric cancer. Polymorphisms in a wide variety of genes, present in a significant proportion of the normal population, may affect the activity of key inflammatory molecules and modify the effect of environmental exposures. Thus, gene-environmental interactions could explain the high inter- individual and/or geographic variations in the gastric cancer incidence. A variety of associations between gastric cancer risk, H. Pylori infection, and specific HLA alleles have been described.

1.6 Precancerous lesions

The secondary prevention focuses on patients at risk of developing gastric cancer. Gastric atrophy, indeed, is considered the first relevant step in the histogenesis of intestinal type gastric cancer according to the multistep process suggested by Correa [28]. In fact, the

risk of gastric cancer is closely related to the grade and extension of gastric atrophy being up to 80-90 folds higher in respect to the general population in patients with severe atrophy involving both antrum and body. Gastric atrophy assumes a precancerous meaning particularly when it is located or extended in the corpus. This latter condition, indeed, damaging parietal cells decreases the acidity in the stomach and provokes the transformation of nitrates food components in nitrites and nitrosamides which are critical for the onset of the gastric carcinogenic process. This hypothesis links the theory of "N-nitroso compounds-mediated gastric cancer risk" with that of "H. pylori-related gastric cancer risk" suggesting an "integrated model" of gastric carcinogenesis. Chronic atrophic gastritis is often associated with intestinal metaplasia, the subsequent step in the Correa model of H. pylori-related gastric carcinogenesis. The prevalence of intestinal metaplasia was significantly higher in H. pylori-positive (43%) than in H. pylori-negative subjects (6.2%). Intestinal metaplasia has been classified according to Jass and Filipe as complete or type I, or incomplete which comprises types II and III. The association between the risk of gastric cancer development and intestinal metaplasia subtypes is, however, not universally accepted. Intestinal metaplasia involving the lesser curvature, from the cardia to the pylorus, or the entire stomach, was associated with a higher risk of gastric cancer than focal or antral predominant intestinal metaplasia. Thus, the distribution of intestinal metaplasia rather than intestinal metaplasia subtype may provide a higher predictive value of cancer risk [29].

The next step in the cascade of morphological changes in gastric carcinogenesis is dysplasia that usually develops in the H. pylori infection, atrophy and intestinal metaplasia setting. The development and progression of dysplastic changes is clearly associated with H. pylori. This process includes a continuum of progressively dedifferentiated phenotypes which may result in a new cell. According to the definition of the World Health Organization, dysplasia is now called non-invasive gastric neoplasia, indicating a pre-invasive neoplastic change in the gastric glands. The higher the grade of dysplasia, the greater the risk of developing invasive gastric cancer. The majority of carcinoma found in follow-up studies and which were discovered within one year of the diagnosis of dysplasia may indicate that the carcinoma was already present at the time of diagnosis of dysplasia [29].

1.7 Cancer microenvironment and angiogenesis

Multiple genetic and epigenetic alterations in oncogenes, tumor suppressor genes, cell cycle regulators, cell adhesion molecules and DNA repair genes, as well as genetic instability and telomerase activation are responsible for tumorigenesis and progression of gastric cancer [30,31,31]. Differences exist in the pathways leading to intestinal and diffuse types of gastric carcinoma. Gastric cancer cells express a wide array of growth factors and cytokines that act via autocrine, paracrine and juxtacrine mechanisms in the tumor microenvironment [31,33]. These complex interactions between tumor cells and stromal cells confer morphogenesis, angiogenesis, invasion and metastasis. Again the expression of these mediators varies depending on the histological subtype.

Angiogenesis is a complex process in which numerous stimulatory and inhibitory signals, such as integrins, angiopoietins, chemokines, oxygen sensors, growth factors, extracellular matrix proteins, and many other molecules are involved. The relationship between the extracellular matrix influencing angiogenesis and the development or prognosis of gastric cancer is not yet well known (Figure 1) [34].

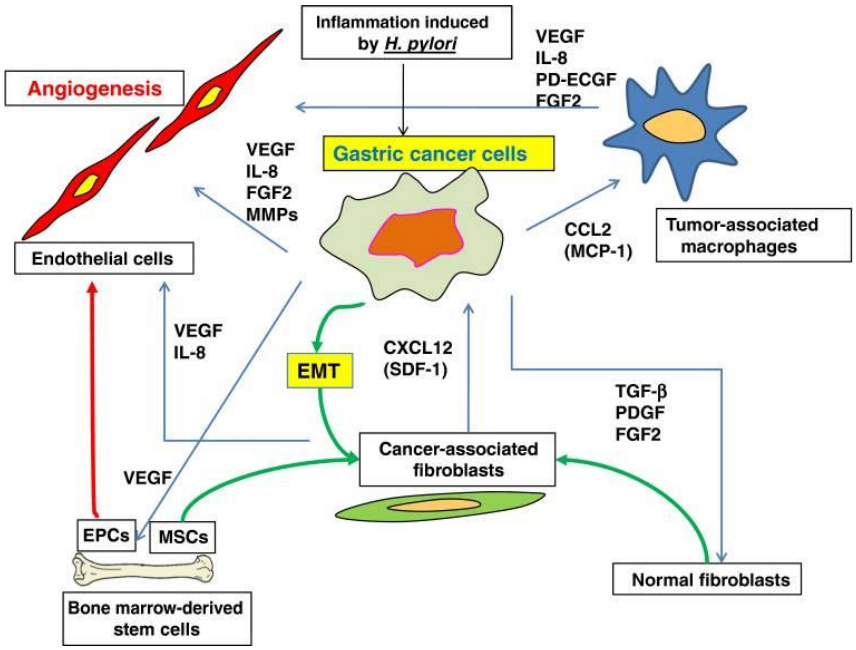

Figure 1. Interaction between gastric cancer cells and stromal cells influences angiogenesis through various angiogenic factors and cytokines. EMT epithelial-to-mesenchymal transition; MSCs mesenchymal stem cells; EPCs endothelial progenitor cells.

Angiogenesis of tumor is mediated by various molecules released by tumor cells and tumor microenvironment [35,36] and gastric cancer cells produce various angiogenic factors, including VEGF [37], CXCL12 [38], IL-8 [39], FGF-2 [40], and platelet-derived endothelial cell growth factor (PD-ECGF) [41].

The CXCL12-CXCR4 pathway has been reported to impact the progression of various malignancies by regulating trafficking of normal and malignant cells [42]. CXCL12-CXCR4 also indirectly promotes tumor metastasis by mediating proliferation and migration of tumor cells and enhancing tumor-associated angiogenesis. The activation of angiogenesis seems to be regulated by pro-inflammatory cytokines, tumor necrosis factor α (TNFα) and interferon γ. CXCL12 gene is located on chromosome 10q 11.1, and it has been revealed that a single nucleotide polymorphism (SNP), a guanine to adenine (G→A), at position 801 of the 3′-untranslated gene region may affect the expression of CXCL12/SDF1 chemokine [43]. The CXCL12/SDF1 A/A homozygotes had been suggested to alter the production of CXCL12/SDF1 and are associated with the risk of carcinogenesis of various origins. Moreover the angiogenic effect of CXCL12 is partly mediated through an induction of Vascular Endothelial Growth Factor (VEGF), suggesting an additive and synergistic mechanism to amplify angiogenesis [42,43,44]. CXCR4 is the chemokine receptor most commonly expressed in tumors and it is the receptor of CXCL12. CXCR4, is located on chromosome 2q2 and a silent SNP (rs2228014) of CXCR4, a cytosine to thymine (C→T), is found at codon 138 [42] and it has been demonstrated to be associated with stages III and IV and also lymph nodes metastasis of oral cancer [45]. The angiogenetic role of CXC12-CXCR4 pathway is confirmed by the neutralizing effect on angiogenesis of antibodies against either CXCL12 or CXCR4 [44].

The first angiogenic chemokine to be described was CXCL8/IL8. IL8 was the first angiogenic chemokine described since 1992. This chemokine is a strong inducer of angiogenesis and also acts as a potent chemoattractant and activator of neutrophils and is closely related to the tumorigenesis, adhesion, invasion and metastasis of cancer. IL8 have been shown to be angiogenic upon interaction with the appropriate chemokine receptors expressed on endothelial cells. Although endothelial cells are characterized by a large degree of heterogeneity dependent upon tissue types, species and vessel caliber, and it is therefore difficult to predict which effect IL8 will be dominant [46]. A well-

characterized SNP at the -251 T/A (rs4073) position of the IL-8 gene has been studied to determine its involvement in several pathologies, including gastric cancer [47,48].

2. WORKING HYPOTHESIS

Early stage gastric cancer, even if surgically curable, maintains a small prevalence of tumor recurrence and, finally, death. For this subgroup, there are increasing bodies of evidence that the best risk stratification for cancer recurrence comes from TNM classification associated with specific molecular prognostic factors. Recent studies have shown that interactions between tumor and stromal cells create a unique microenvironment, essential for tumor growth and metastasis. Chemokines affect tumor development indirectly by influencing angiogenesis, tumor leukocyte interactions, as well as directly by influencing tumor transformation, survival and growth, invasion and metastasis.

Our working hypothesis started from the interesting role of the genetic polymorphisms of CXCR4 and CXCL12 axis and IL8 on the prognosis of various types of solid tumors, and from the lack of the literature on their association with gastric cancer.

3. MATHERIALS AND METHODS

From the large biobank of Clinica Chirurgica I (University of Padova, Italy) 333 patients affected with gastric cancer at different TNM stages of disease (I- IV) who underwent radical surgery since 1991 were selected. Before surgery, and after informed consent, a sample of peripheral blood was withdrawn from each patient and stored. From each sample PBMC (Peripheral Blood Mononuclear Cells) were used for DNA (germline) isolation for allelic discrimination assay.

Variables object of study were: gender, age, overall survival (expressed in months), stage, TNM classification, lymphatic and venous invasion, grading, lymph node metastasis. This study evaluates a set of polymorphisms of three different genes coding for CXCL12 and IL8 (cytokines) and CXCR4 (receptor) and the partial results concerning the following three polymorphisms: rs1801157, rs2228014 e rs4073 (Table 2).

GENE	ID POLYMORPHISM	ALLELIC VARINCES
CXCL12 (SDF1) Homo sapiens chemokine (C-X-C motif) ligand 12, transcript variant 2, chromosome 10	rs1801157	CXCL12-801GG CXCL12-801AA CXCL12-801G/A
CXCR4 Homo sapiens chemokine (C-X-C motif) receptor 4, chromosome 2	rs2228014	CXCR4-138TT CXCR4-138CC CXCR4-138C/T
IL-8 chromosome 4	rs4073	IL8-251AA IL8-251TT IL8-251A/T

Table 2. Polymorphisms object of study

Genomic DNA was extracted by QIAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacture's instructions. DNA was dissolved in TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA] and then quantitated by a measurement of OD_{260}. Final preparation was stored at −20 °C and used as templates for PCR.

The CXCL12-3′A and CXCR4 polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The sequences of primers used to amplify the CXCL12-3′A genotype were 5′-CAGTCAACCTGGGCAAA GCC-3′ and 5′-CCTGAGAGTCCTTTTGCGGG-3′, and those used for the amplification of CXCR4 genotype were 5′-AACTTCCTATGCAAGGCAGT-3′ and 5′-TATCTGTCATCTGCCTCACT-3′. PCR was performed in a 10 μL reaction mixture containing 100 ng DNA template, 1.0 μL of 10× PCR buffer (Invitrogen, Carslbad, CA), 0.25 U of Taq DNA polymerase (Invitrogen, Carslbad, CA), 0.2 mM dNTPs (Promega, Madison, WI), and 200 nM of each primer (MDBio, Taipei, Taiwan). The PCR cycling started at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, with a final step at 72 °C for 20 min to allow a complete extension of all PCR fragments. PCR products of CXCL12 and CXCR4 gene were subjected to enzymatic digestion by incubation with HpaII and BccI for 4 h at 37 °C and then submitted to electrophoresis in 3% agarose gels. For CXCL121, wild type homozygous alleles (G/G) yielded 100 and 193-bp products, the heterozygous alleles (G/A) yielded 100-, 193- and 293-bp products, while the mutated type homozygous alleles (A/A) yielded a 293-bp product. For CXCR4 gene, wild-type homozygous alleles (C/C) yielded 103 and 133-bp products, the heterozygous alleles (C/T) yielded 103-, 133- and 236-bp products, while the mutated type homozygous alleles (T/T) yielded a 236-bp product. Digested RFLP products were electrophoresed in 2 % agarose gels, stained with ethidium bromide, visualized under ultraviolet light, and photodocumented.

The IL8 polymorphisms were determined by PCR-RFLP assay. The sequences of primers used to amplify IL8 genotype were forward primer 5′-CATGATAGCATCTGTAATTAACTG-3′ and reverse primer 5′-CTCATCTTT TCATTATGTCAGAG-3′. PCR conditions involved an initial denaturation step of 94 °C 5 min, followed by 35 cycles of 94 °C for 45 s and 52 °C for 45 s. Then, another cycle of 72 °C for 7 min was carried out before termination. Ten microliters of the PCR product was digested after incubation for 60 min at 37 °C with 0.5 U of the restriction enzyme

MfeI (New England BioLabs, Ipswich, MA, USA). To analyze the polymorphism, gel electrophoresis was performed on the digested PCR products; the AA homozygotes yielded bands of 202 bp and 147 bp, the TT wild type yielded one band of 349 bp, and the TA heterozygotes yielded three bands of 349 bp, 202 bp, and 147 bp. Digested RFLP products were electrophoresed in 2 % agarose gels, stained with ethidium bromide, visualized under ultraviolet light, and photodocumented.

Statistical analysis was performed with STATA 11.2 SE (StataCorp, College Station TX, USA). Cox regression univariate analysis was performed for overall survival, and Kaplan-Meier method for survival curves. Multivariate statistical analysis was performed to test a correlation between the polymorphisms objet of study and the variables considered of interest. P-values less than 0.05 were considered as significant.

4. RESULTS

In this study we enrolled 333 patients (155 female and 178 male) with a median age of 64,7 years (range 28-90). The mean overall survival calculated was 52 months (134 patients still alive, 186 non alive). Staging for gastric cancer revealed 155 patients (46%) with early stage gastric cancer (stage I-II), 53 patients (16%) with stage III and 115 patients (34%) with stage IV (Figure 1). Lymph node were free from metastasis in 100 patients, and metastatic in 211 patients. Distant metastasis were present in 71 patients and absent in 261 patients.

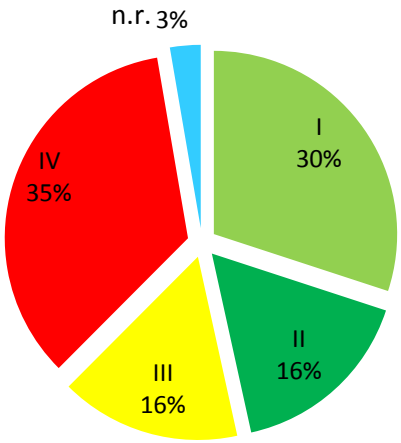

Figure 1. Cancer stage
(n.r. = undetermined)

Patient's stratification for genetic polymorphisms are summarized in Figure 2, Figure 3 and Figure 4.

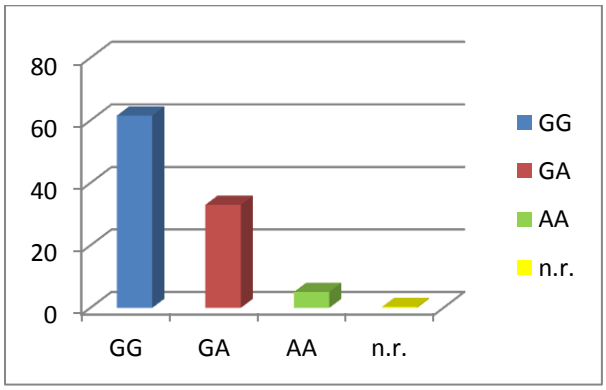

Figure 2. CXCL12 stratification according to genotypes. (n.r.= undetected).

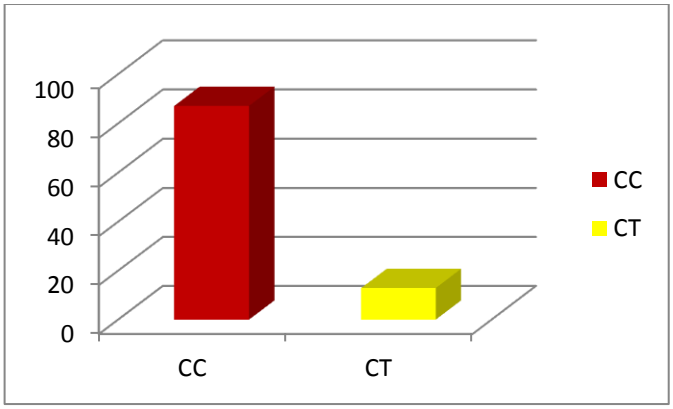

Figure 3. CXCR4 stratification according to genotypes.

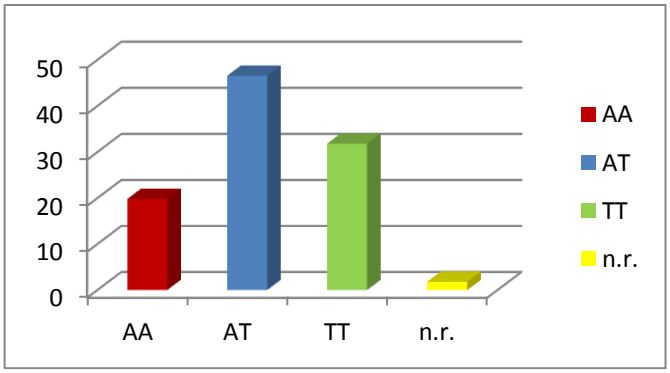

Figure 4. IL8 stratification according to genotypes. (n.r. = undetermined).

Survival analysis reported no statistically significant results (p-value > 0.05) in allelic, dominant and recessive, genetic models.

Grading analysis reported no statistically significant results (p-value > 0.05) in allelic, dominant and recessive, genetic models.

Venous infiltration analysis reported no statistically significant results (p-value > 0.05) in allelic, dominant and recessive, genetic models.

Lymphatic infiltration reported no statistically significant results (p-value > 0.05) in allelic, dominant and recessive, genetic models.

Lymph node invasion analysis reported no statistically significant results (p-value > 0.05) in allelic, dominant and recessive, genetic models for CXCL12 and CRCR4. Instead, a

weak association (p-value = 0.049) between genotypes A/A and A/T of rs4073 of the gene encoding for IL8 and lymph node involvement was found in the dominant model.

The logistic regression performed for lymph node invasion as dependent variable and TT polymorphism as independent variable revealed an odds ratio of 0.60, p-value of 0.50 and a 95% confidence interval of 0.363-0.999; suggesting an association of the TT allele with a decrease risk to develop lymph node metastasis of 40%. However, at multivariate analysis, TT polymorphism was not correlated with T (primary tumor of TNM classification), so rs4073 seems not to ameliorate the information given by the T of the TNM classification for lymph node invasion.

5. DISCUSSION

Gastric cancer and early stage gastric cancer, even if surgically curable, maintains a small prevalence of tumor recurrence and, finally, death. There are increasing bodies of evidence that the best risk stratification for cancer recurrence comes from TNM classification associated with specific molecular prognostic factors. Recent studies have shown that interactions between tumor and stromal cells create a unique microenvironment, essential for tumor growth and metastasis [49]. Chemokines affect tumor development indirectly by influencing angiogenesis, tumor leukocyte interactions, as well as directly by influencing tumor transformation, survival and growth, invasion and metastasis [50]. Metastasis is a frequent cause of death in patients with gastric cancer and can often occur after surgery, with absent or minimal lymph node involvement and without intra-operative macroscopic signs of peritoneal carcinomatosis [43]. Even early stages are at risk: once tumor cells have spread through the mucosa infiltration level 3 (m3) and to the submucosa, the probability of metastasis increases rapidly and the likelihood of prolonged disease-free survival diminishes [51]. Thus, it is of major importance to identify those patients with a high risk for metastatic disease. Apart from hematogenous invasion in organ metastasis, peritoneal carcinomatosis of gastric carcinoma may develop from direct cancer cell dissemination into the abdominal cavity. The expression of chemokine receptors by tumor cells can be an important factor with regard to tumor cell dissemination and organ-specific metastases [52-54].

The CXCL12-CXCR4 pathway has been reported to impact the progression of various malignancies by regulating trafficking of normal and malignant cells [34]. CXCL12-CXCR4 also indirectly promotes tumor metastasis by mediating proliferation and migration of tumor cells and enhancing tumor-associated angiogenesis. The activation of angiogenesis seems to be regulated by pro-inflammatory cytokines, tumor necrosis factor α (TNFα) and interferon γ. Moreover the angiogenic effect of CXCL12, which is expressed in the lung and other sites of metastasis, is partly mediated through an induction of Vascular Endothelial Growth Factor (VEGF), suggesting an additive and synergistic mechanism to amplify angiogenesis [42]. CXCR4 is the chemokine receptor most commonly expressed in tumors and it is the receptor of CXCL12. The angiogenetic role of CXC12-CXCR4 pathway is confirmed by the neutralizing effect on angiogenesis of antibodies against either CXCL12 or CXCR4.

IL8 is a strong inducer of angiogenesis and also acts as a potent chemoattractant and activator of neutrophils and is closely related to the tumorigenesis, adhesion, invasion and metastasis of cancer [55]. IL8 have been shown to be angiogenic upon interaction with the appropriate chemokine receptors expressed on endothelial cells. Although endothelial cells are characterized by a large degree of heterogeneity dependent upon tissue types, species and vessel caliber, and it is therefore difficult to predict which effect IL8 will be dominant.

In our study CXCL12, CXCL4 and IL8 polymorphisms did not impact overall survival of patients who underwent surgery for curative intent. A multivariate analysis was performed to identify correlation between the polymorphisms and gastric cancer grading, venous or lymphatic infiltration and lymph nodes metastasis.

As shown in other studies [56-59], we observed an increased risk of lymph node metastasis in patients expressing A/A and A/T of rs4073 in the promoter region of the gene encoding for IL8. Even if the support from previous literature, we did not observed the expected results: no independent prognostic factor was found.

6. STUDY LIMITS

We started our work from the idea that cancer microenvironment may have influenced the survival of our gastric cancer patients, so we focused on poorly studied angiogenetic factors (CXCL12, CRCR4 and IL8).

The first problem we encountered was the lack of follow up data. Our retrospective observational study enrolled patients since 1992 so it was virtually impossible to understand the real cause of death. This is the reason why we calculated overall survival and not specific survival.

Another limit is that we didn't study for SNPs control subjects yet, but this is one of the next target.

Patient's population is not enough to support rs1801157, rs2228014, rs4073 as independent prognostic factors for gastric cancer and in particular early stage gastric cancer.

6. CONCLUSIONS

In conclusion our preliminary results are not conclusive and further studies are needed to clarify the intriguing role of angiogenetic cytokines polymorphisms and their role in the guide of lymph node metastatization and distant metastatization.
A prospective case-control study is the next postdoc research topic.

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